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The effect of beta-2 adrenoreceptor agonist inhalation on lungs donated after cardiac death in a canine lung transplantation model

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Abbreviations

ADP: adenosine diphosphate

AMP: adenosine monophosphate

ANOVA: analysis of variance

AoP: aortic pressure

ATP: adenosine triphosphate

cAMP: cyclic adenosine monophosphate

CO: cardiac output

CVP: central venous pressure

DCD: donation after cardiac death

ELISA: enzyme-linked immunosorbent assay

IL: interleukin

IRI: ischemia reperfusion injury

LAP: left atrial pressure

MPO: myeloperoxidase

PAP: pulmonary artery pressure

TNF: tumor necrosis factor

WDR: wet-to-dry weight ratio

WIT: warm ischemic time

Abstract

Background: It is a matter of great importance in a donation after cardiac death to attenuate ischemia reperfusion injury (IRI) related to the inevitable warm ischemic time. **Methods:** Donor dogs were rendered cardiac-dead and left at room temperature. The dogs were allocated into two groups: the beta-2 group (n = 5) received an aerosolized beta-2 adrenoreceptor agonist (procaterol 350µg) and ventilation with 100% oxygen for 60 min starting at 240 min after cardiac arrest, and the control group (n = 6) received an aerosolized control solvent with the ventilation. Lungs were recovered 300 min after cardiac arrest. The recipient dogs underwent left single lung transplantation to evaluate the functions of the left transplanted lung for 240 min after the reperfusion. **Results:** In the beta-2 group, the oxygenation and dynamic compliance were significantly higher than the control group. The beta-2 group revealed significantly higher levels of cyclic adenosine monophosphates and high-energy phosphates in the donor lung after the inhalation compared to before it. Furthermore, the histological findings revealed that the beta-2 group had less edema and fewer inflammatory cells.

Conclusion: Our results suggest that beta-2 adrenoreceptor agonist inhalation during the pre-procurement period may ameliorate IRI.

Key words: beta-2 adrenoreceptor agonist, canine model, donation after cardiac death, inhalation, lung transplantation

Introduction

One method of countering chronic lung donor shortages is the practice of donation after cardiac death (DCD). This technique inevitably leads to ischemia reperfusion injury (IRI) related to warm ischemia. However, DCD is now increasingly accepted in some countries.

Beta-2 agonists in humans, injected intravenously or aerosolized and administered through the airway, were reported to have protective effects against high altitude lung edema ¹, ARDS ², pulmonary edema after lung resection ³ and pulmonary edema of discarded lungs from brain-dead donors in an *ex vivo* model ⁴. Moreover, beta-2 agonists have already been reported to have protective effects against IRI in rat models, including a study by our group ⁵. Therefore, we hypothesized that a beta-2 adrenoreceptor agonist would ameliorate IRI in our canine model.

Materials and Methods

Animals

Eleven pairs of weight-matched TOYO Beagles (8.55-10.65 kg, Kitayama Labes Co. Ltd., Hongo Farm, Yamaguchi, Japan) were used in this study. All animals received humane care in compliance with the *Principles of Laboratory Animal Care*, formulated by the National Society for Medical Research, and the *Guide for the Care and Use of Laboratory Animals*, prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996). The study protocol was approved by the ethics committee of the Graduate School of Medicine at Kyoto University, Japan.

Donor preparation

Donor dogs were anesthetized via an intramuscular injection of midazolam (0.5 mg/kg), xylazine (0.25 mg/kg), and atropine sulfate (0.05 mg/kg). They were then intubated and mechanically ventilated at a tidal volume of 25 ml/kg and a rate of 15 breaths/min. The positive end-expiratory pressure was 5.0 cm H₂O;

the inspired O₂ fraction was 1.0. They were euthanized via the intravenous administration of potassium chloride (0.5 mEq/kg), without heparinization. The ventilators were removed, and the tracheal tubes were left open to room air. All donors were left at room temperature (21°C) for 300 min. The beta-2 group (n = 5) received inhalation of the beta-2 adrenoreceptor agonist (3.5ml of 0.01% pocaterol inhalation solution (350µg of procaterol)) during ventilation with 100% oxygen for 60 min starting at 240 min after cardiac arrest. The control group (n = 6) receive inhalation of the control solvent (3.5ml) with the same ventilation setting as the beta-2 group. The inhalation solution and the control solvent were aerosolized by a nebulizer (Aeroneb Professional Nebulizer System, Aerogen, Ireland) which was inserted into the inspiratory loop of a ventilator. A pulmonary arterial flush was performed on all donors at 300 min after the cardiac arrest, using ET-Kyoto solution (Otsuka Pharmaceutical Co. Ltd. Tokyo, Japan), an extracellular preservation solution that we originally developed for clinical lung transplantation ⁶. Prior to use, the ET-Kyoto solution was stored at 4°C without heparin. For the antegrade flush, we used 100 ml/kg; 50 ml/kg was used for the

retrograde flush. Both were performed from 30 cm above the donors' chests.

Ventilation was performed during the flush. The recovered organs, semi-inflated (within 20 cm H₂O) with 100% oxygen, were preserved at 4°C for 120 min.

Recipient preparation and transplantation

Recipient dogs were anesthetized, maintained, and ventilated in the same manner as the donors. Peak inspiratory pressure was monitored by a pressure transducer that was attached to the tracheal tube. For each recipient, a 5F thermodilution catheter (151F7, Edwards Lifesciences, Irvine, CA, USA) was placed in the main pulmonary artery from the right femoral vein to measure pulmonary artery pressure (PAP), central venous pressure (CVP), and cardiac output (CO). A femoral arterial line was inserted for measuring aortic pressure (AoP) and for arterial blood gas analysis. After thoracotomy, a 2.0-mm catheter was inserted directly into the left atrium for monitoring left arterial pressure (LAP). We then performed a left pneumonectomy and left lung transplantation. The right pulmonary artery was encircled with vascular tape and clamped with a tourniquet

continuously from 60 min to 240 min after reperfusion in order to evaluate the function of the transplanted left lung.

Definitions

The warm ischemic time (300 min) was defined as the time from cardiac arrest to the pulmonary arterial flush. The cold ischemic time (120 min) was defined as the time from the initiation of the flush of the donor lung until the lung was removed from cold storage for transplantation. The transplantation time (60 min) was defined as the time from the removal of the lung from storage to the reperfusion after we performed transplantation. Therefore, the total ischemic time was defined as the sum, 480 min.

Assessment of the lungs and their function

Arterial and pulmonary arterial blood samples were taken in order to analyze the blood gases with a blood gas analyzer (iSTAT Portable Clinical Analyzer, iSTAT Corp., East Windsor, NJ, USA).

Wet-to-dry lung weight ratio

Left lung tissues (100-300 mg) were used to calculate the wet-to-dry weight ratio (WDR) at 240 min after reperfusion or at death. The tissues were excised from the tip of the lung and the cut end was closed by ligation. Wet weight (mg) was measured first; dry weight (mg) was measured after the tissue had been dried overnight at 180°C. The WDR was calculated by dividing the wet weight by the dry weight.

Measurement of adenine nucleotide levels

We used pieces of peripheral lung tissues collected from “normal lungs”, which were collected from the left lungs of recipients just after the induction of anesthesia. Furthermore, we used samples from donors before and after ventilation and from recipients at 240 min after reperfusion. We measured the levels of adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) using high-performance liquid

chromatography (Shim-pack CLC-ODS column; 15 cm × 6.0 mm; Shimadzu, Japan). Total adenosine nucleotides (TAN) and the energy charge were calculated as previously reported ⁵.

Measurement of cAMP levels

We used pieces of the peripheral lung tissues from donors before and after ventilation to determine the cAMP levels. The levels of cAMP were measured with a radioimmunoassay kit (cAMP assay kit; Yamasa, Chiba, Japan) as previously reported ⁵. At the same time, protein levels were measured according to the method described by Lowry and coworkers ⁷.

The macroscopic findings and histology (hematoxylin and eosin staining)

We recorded the macroscopic appearances of the left lungs 240 min after reperfusion. Left lung specimens collected 240 min after reperfusion were also used for the histological analysis. Each lung was immersed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin.

Naphthol AS-D chloroacetate esterase staining

The accumulation of activated neutrophils in paraffin-embedded sections of left lungs harvested at 240 min after reperfusion was assessed by staining for chloroacetate esterase, a neutrophil-specific marker, according to the method described by Leder⁸. Polymorphonuclear cells, identified by their staining and morphology, were counted in 10 randomly chosen high-power fields per section at an original magnification of 400x. Three separate investigators (J.S., F.C., and D.N.) evaluated, interpreted, and reached a consensus, without any knowledge of the experimental groups.

Myeloperoxidase activity

The level of myeloperoxidase (MPO) was measured in the peripheral lung tissues collected in recipients at 240 minutes after reperfusion. We followed the instructions in the manufacturer's manuals from the MPO Assay Kit (BioVision Research Products, Mountain View, CA, USA).

Serum cytokine levels

We used enzyme-linked immunosorbent assays (ELISA) to measure the serum IL-6, IL-8, and TNF- α levels at 240 min after reperfusion in surviving animals. In all cases, we followed the instructions in the manufacturer's manuals from the IL-6, IL-8, and TNF- α ELISA kits (Quantikine, R&D Systems Inc., Minneapolis, MN, USA).

Statistical analyses

All data were analyzed using the JMP[®] 9 software program (SAS Institute Inc., Cary, NC, USA). A paired *t*-test was used to compare the pre-ventilation and post-ventilation samples with or without the beta-2 agonist inhalation. An unpaired *t*-test or a repeated-measures analysis of variance (ANOVA) was used to explore the differences between and within the groups. The ANOVA was performed between 75 and 240 min in order to evaluate only the transplanted left lungs after the clamp of right pulmonary artery. Significance was defined as $P <$

0.05.

Results

There were no significant differences between the treatment groups in terms of the donor or recipient weights (the beta-2 group vs. the control group, donor weight: 10.3 ± 0.2 vs. 10.1 ± 0.3 kg; recipient weight: 9.2 ± 0.4 vs. 9.3 ± 0.2 kg).

We set 60 min for the implantation time in our protocol, and were able to perform all transplants within 60 min. All 5 animals in the beta-2 group survived until the end-point of the study (240 min after reperfusion), while 5 out of 6 animals in the control group survived. The dead animal expired as the result of graft dysfunction between 75 and 120 min after reperfusion.

Assessment of lung function

The PaO_2 at 240 min after reperfusion was 526.8 ± 130.2 vs. 112.8 ± 87.3 mmHg

for the beta-2 group vs. the control group. Furthermore, by a repeated analysis of variance between groups, the PaO₂ and dynamic compliance were found to be significantly higher in the beta-2 group than in the control group ($P < 0.001$ and $P = 0.013$ respectively; Table 1). The cardiac output was lower and pulmonary vascular resistance was higher in the control group, but this difference did not reach significance ($P = 0.087$ and 0.086 respectively Table 1).

Wet-to-dry lung weight ratio

The WDR in the control group was generally higher than that in the beta-2 group at 240 min after reperfusion, but the difference was not significant (7.0 ± 2.0 vs. 9.23 ± 1.95 , respectively; $P = 0.11$).

Measurement of adenine nucleotide levels

The control solvent inhalation did not increase the ATP levels in the control group (Figure 1A), while, in the beta-2 group, the ATP levels were significantly improved after the 60 min inhalation compared to before the inhalation ($P =$

0.027; Figure 1B), Moreover, at 240 min after reperfusion, the ATP and TAN (TAN = ATP + ADP +AMP) levels were significantly higher in the recipient lungs of the beta-2 group than in the recipient lungs of the control group ($P= 0.012$ and 0.027 , respectively; Table 2B), and the ATP and TAN levels improved similarly to those in the normal lungs (Table 2A).

Measurement of cAMP levels

The control solvent inhalation did not increase the cAMP levels in the control group (Fig. 1C), while, in the beta-2 group, the cAMP levels were significantly improved after the 60 min inhalation compared to the levels before the inhalation ($P = 0.045$; Figure 1D).

Macroscopic findings and histology

At 240 min after reperfusion, the lungs from the control group macroscopically had dark-red areas exhibiting more patchiness than was observed in the beta-2 group (Figures 2A, B). The histological study indicated severe intraparenchymal

hemorrhaging and inflammation only in the control group (Figure 2C) in contrast to the beta-2 group (Figure 2D).

Naphthol AS-D chloroacetate esterase staining

At 240 min after reperfusion, there were significantly more polymorphonuclear cells in the lungs of the control group (Figure 2E) than in the beta-2 group (Figure 2F) ($P < 0.001$; Figure 2G).

MPO activity

At 240 min after reperfusion, the MPO activity levels in lung tissues were significantly higher in the control group than in the beta-2 group ($P = 0.0058$; Figure 3A).

Serum cytokine levels

At 240 min after reperfusion, the serum TNF- α levels were significantly higher in the control group than in the beta-2 group ($P = 0.011$; Figure 3B), while the IL-6

and IL-8 levels had no significant difference (Figures 3C, D).

Discussion

In this study, we found that beta-2 adrenoreceptor agonist inhalation ameliorated ischemia reperfusion injury in our canine model, confirming and expanding our previous study in rats ⁵.

Beta-2 adrenoreceptors were reported to be distributed in alveolar cells, airway epithelium, airway smooth muscle, and pulmonary vessels in humans ⁹, which suggested that inhaled beta-2 agonists might have actions on various pulmonary tissues, especially with regard to relaxing the airway and vessels. Moreover, beta-2 agonists was reported to cause vasodilation and increase the bronchial blood flow, which arises from the aorta and forms a peribronchial plexus of vessels which is part of the systemic circulation ¹⁰. In addition, the beta-2 inotropic effect of the agonist significantly increased the cardiac output and pulmonary artery pressure, while decreasing the pulmonary vascular

resistance ¹¹. These reports may coincide with our findings, wherein we detected significantly higher dynamic compliance, relatively higher level of cardiac output ($P = 0.087$) and relatively lower level of pulmonary vascular resistance ($P = 0.086$) in the beta-2 group, although we didn't evaluate the compliance of only the transplanted left lungs because we didn't clamp the right bronchus.

The mechanism by which the inhalation provides protection can be inferred from our measurements of the cAMP levels, adenine nucleotide levels, and inflammatory cells and cytokines. First, we found that the cAMP levels were significantly increased between the measurements taken before and after the inhalation. Beta-2 adrenoreceptor agonists were reported to decrease capillary permeability by increasing cAMP ¹² as a result of tighter intercellular junctions and actomyosin myofibrils relaxation ¹³, thereby decreasing the lung edema.

Second, the adenosine nucleotide levels recorded herein suggest that the beta-2 agonist inhalation increased the energy TAN and ATP levels in the donor lungs, as well as the ATP and TAN levels in the recipients. A previous report revealed that increased TAN levels have been significantly associated

with lung viability ¹⁴.

Third, infiltrating leukocytes are a major factor involved in IRI ¹⁵. In the current study, polymorphonuclear cells with AS-D staining were less prevalent, and the MPO activity was lower in the beta-2 group. These findings coincide with reports that the systemic administration and inhalation of beta-2 adrenoreceptor agonists diminished neutrophil recruitment to the lungs of experimental animals. In terms of the mechanism, diminished intercellular cAMP in the endothelial cells during hypoxia is known to increase leukocyte adhesion ¹⁶, and cAMP negatively regulates the migratory capacity of neutrophils ¹⁷. Our findings of higher cAMP levels in the beta-2 group may therefore coincide with these mechanisms.

Fourth, we observed significant decreases in the serum levels of the inflammatory cytokine TNF- α . In previous reports, the systemic administration of several beta adrenoreceptor agonists potently inhibited TNF- α during endotoxemia¹⁸, and inhaled salmeterol reduced TNF- α in the BALF after inhalation of lipopolysaccharide in humans ¹⁹. Our finding showing a reduced serum TNF- α level suggests that beta-2 adrenoreceptor agonist inhalation may

reduce the inflammatory change in uncontrolled DCD donors.

One limitation of our study is that we did not examine an adequate dose of procaterol in our model. Our dose, 350 µg, was likely too high, because 30 to 50µg is clinically prescribed for one inhalation of an adult. However, frequent inhalation (~500µg/day) of procaterol in younger and elder adults was reported to be safe ²⁰. Furthermore, we used high doses of medication in DCD donors 240 min after cardiac arrest, which meant that there was enough long time after the declaration of death from the ethical aspect. Therefore, in our experiments, we selected the dose of procaterol based on the likely maximum dose that could be used for the 60 min ventilation.

The beta-2 adrenoreceptor agonist inhalation has not been routinely performed yet in brain-dead donors in Japan. One of the reasons is that it is difficult to change the law concerning the management of brain-dead donors in Japan. Now, our group has revealed the effect of beta-2 agonist inhalation in the experimental DCD in rat⁵ and this canine model. Therefore, these results would strongly encourage clinicians to conduct beta-2 adrenoreceptor agonist

inhalation in brain-dead donors and, in the near future, DCD donors.

In conclusion, we found that the protective effect of beta-2 adrenoreceptor agonist inhalation during the last 60 min of a 300 min WIT preserves the lung function of DCD donors. Therefore, the use of the beta-2 adrenoreceptor agonist inhalation during this period should be conducted in a clinical setting of DCD.

Disclosure statement

Procaterol inhalation solutions were purchased from Otsuka Pharmaceutical Co. Ltd. and the control solvents were provided by the company free of charge without any restrictions as to the nature of the research.

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of the presented manuscript or other conflicts of interest to disclosure.

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Figure Legends

Figure 1. The ATP and cAMP levels before (pre-vent.) and after (post-vent.) the inhalation in donor lungs. Both the ATP and cAMP levels did not change significantly in the control group (A, C), while they increased significantly in the beta-2 group (B, D), as determined using a paired *t*-test.

Figure 2. The results of the macroscopic (A, B) and histological (C-F) analyses conducted 240 min after reperfusion (hematoxylin-eosin staining(C, D), original magnification x 100 and naphthol AS-D chloroacetate esterase staining (E, F), x 400). In the control group, we observed many dark-red spots (A) and alveolar congestion, hemorrhage, infiltration, aggregation of inflammatory cells, and edema (C). However, the lungs in the beta-2 group looked relatively normal (B, D). In naphthol AS-D chloroacetate esterase staining, subjects in the control group (E) possessed significantly higher numbers of polymorphonuclear cells than subjects in the ventilation group (F). The data are presented as the means \pm SD and were analyzed using an unpaired *t*-test (G).

Figure 3. The myeloperoxidase activity of lung tissues (A) and the serum cytokine levels (B-D) in the control and beta-2 groups 240 min after reperfusion.

The myeloperoxidase activity (A) and TNF- α (B) were significantly higher in the control group. The data are presented as the means \pm SD and were analyzed using an unpaired *t*-test.

Figure 1.

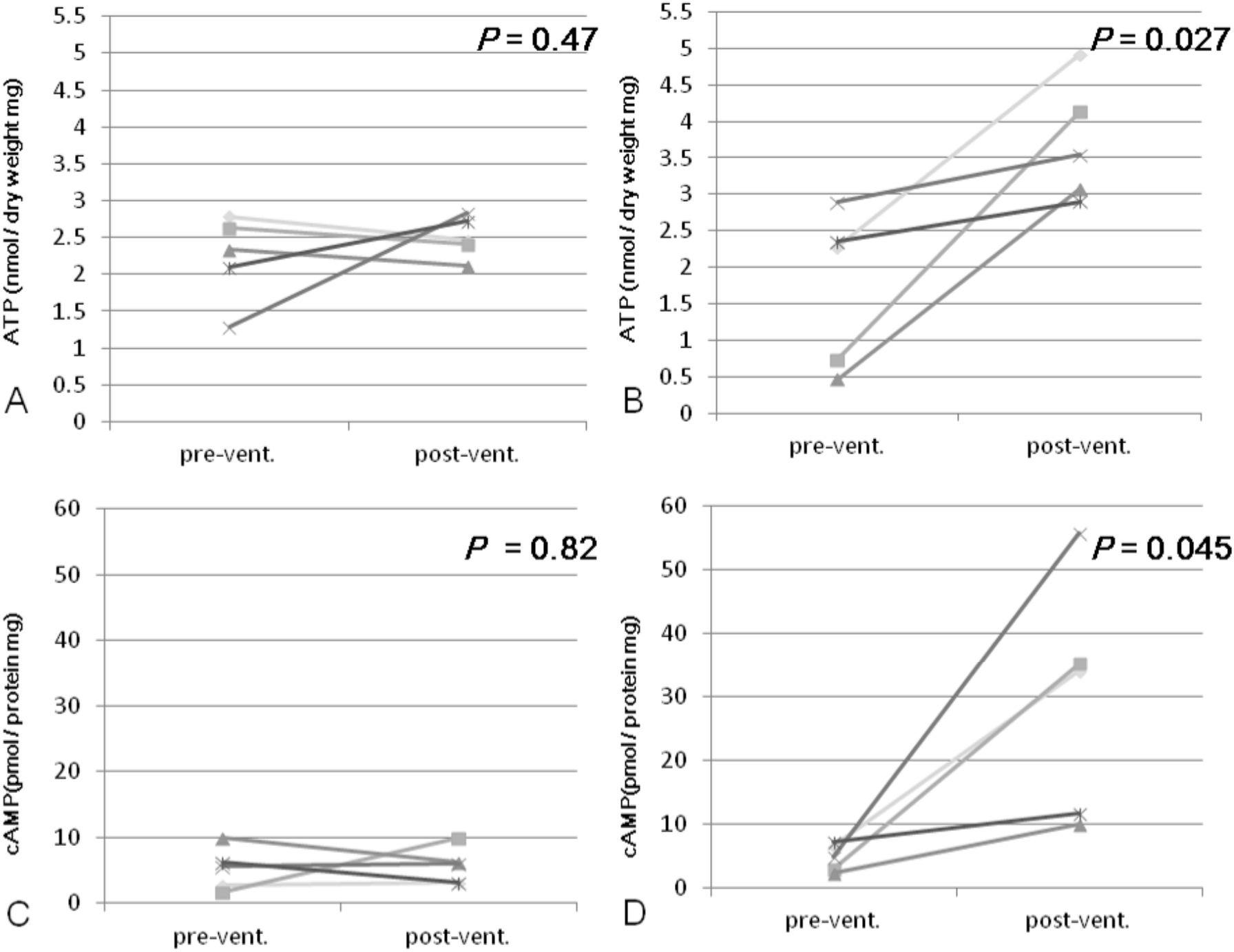
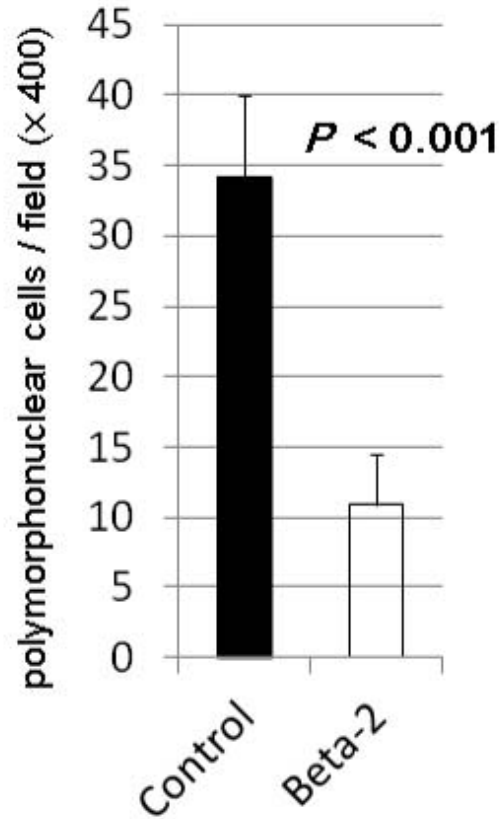
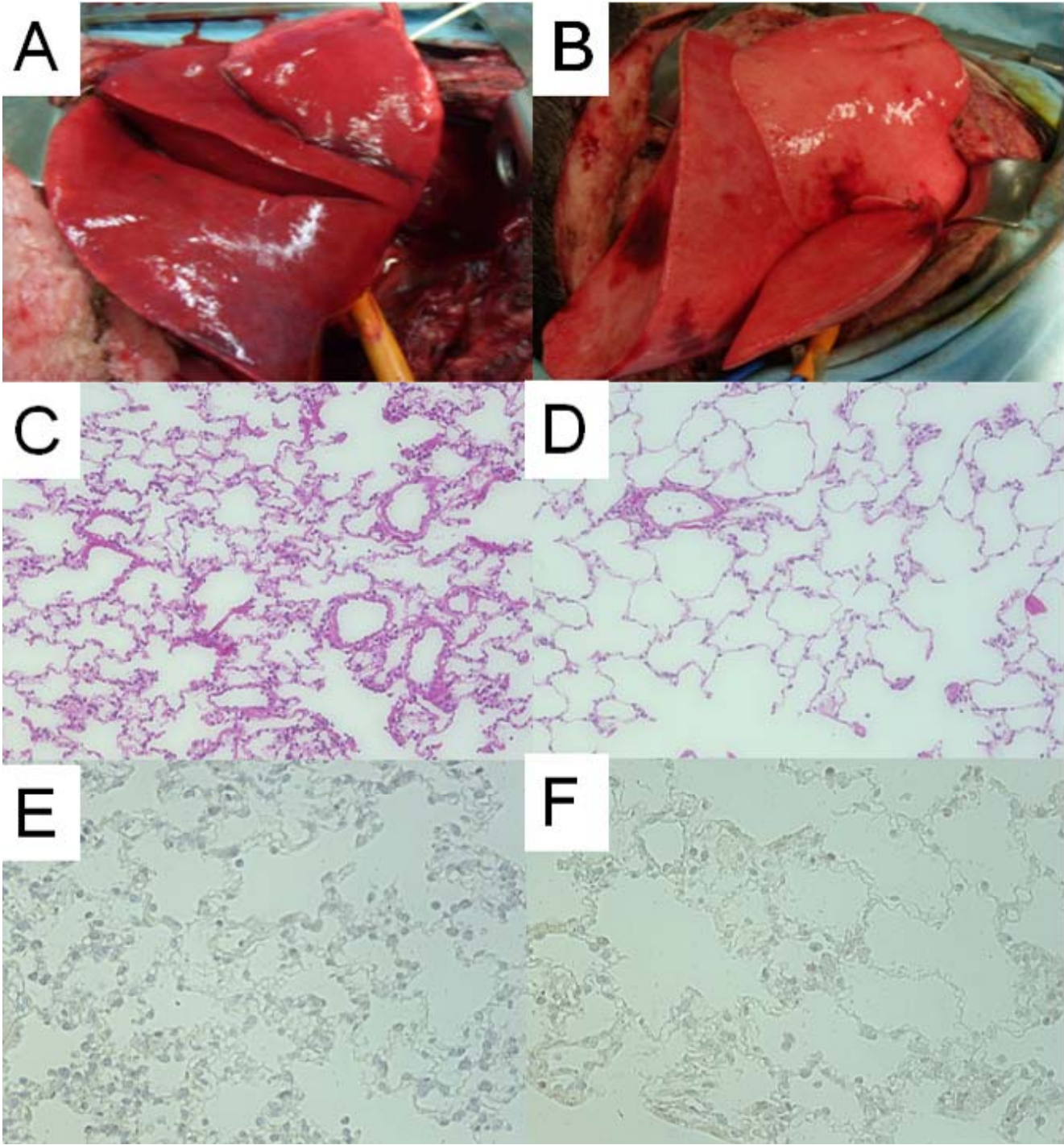


Figure 2.



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Figure 3.

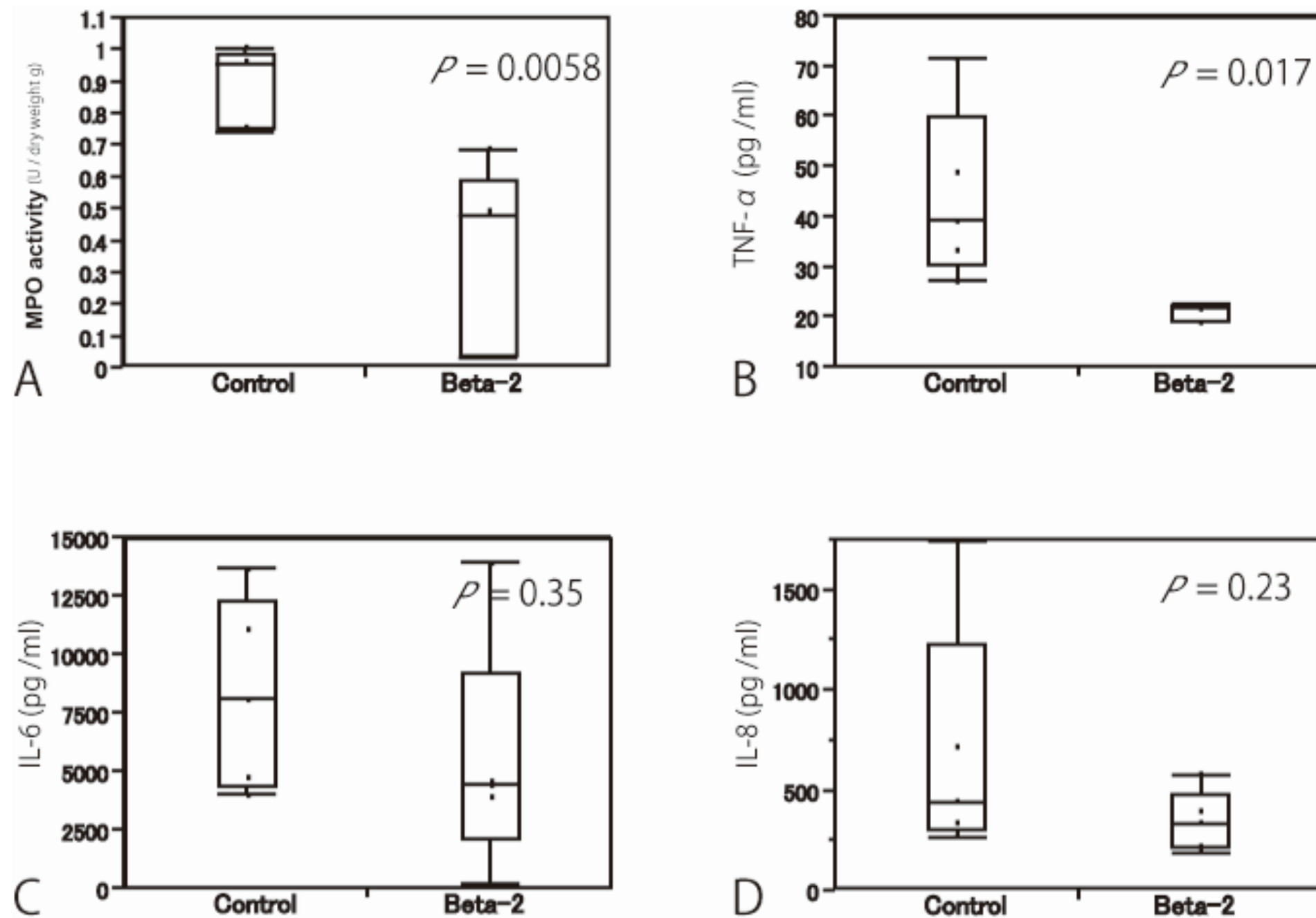


Table 1. Assessment of lung function in surviving recipients in the control (n = 5) and beta-2 (n = 5) groups.

Time after transplantation, min						
	(Before OP)	75	120	180	240	<i>P</i> -value
Mean AoP, mmHg						
Control	(123.2 ± 27.2)	98.0 ± 27.2	110.8 ± 26.6	86.6 ± 24.0	65.0 ± 38.8	0.17
Beta-2	(114.6 ± 17.2)	102.0 ± 12.3	124.0 ± 13.4	118.2 ± 26.8	94.8 ± 36.5	
Mean PAP, mmHg						
Control	(17.6 ± 2.0)	40.0 ± 11.7	41.8 ± 7.8	40.8 ± 6.7	27.0 ± 12.3	0.87
Beta-2	(19.8 ± 2.1)	39.2 ± 14.4	38.2 ± 16.1	38.8 ± 16.2	37.6 ± 16.2	
Mean CVP, mmHg						
Control	(5.6 ± 2.0)	6.4 ± 2.0	6.0 ± 2.5	6.8 ± 1.6	8.6 ± 4.7	0.47
Beta-2	(5.4 ± 2.3)	9.0 ± 3.6	8.2 ± 3.4	8.0 ± 2.5	7.8 ± 3.5	
Mean LAP, mmHg						
Control	(11.8 ± 1.9)	6.0 ± 1.8	5.2 ± 1.9	7.4 ± 2.3	7.4 ± 1.1	0.10
Beta-2	(11.2 ± 2.7)	13.6 ± 7.4	10.8 ± 5.0	11.0 ± 6.5	10.2 ± 5.0	
Cardiac output, l/min						
Control	(0.8 ± 0.2)	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.5 ± 0.3	0.087
Beta-2	(0.8 ± 0.2)	1.1 ± 0.4	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.2	
PVR, dyn·s·cm ⁻⁵ x 10 ³						
Control	(569.4 ± 76.3)	4292.4 ± 1915.0	3844.8 ± 1212.7	3415.1 ± 1160.8	2717.3 ± 844.1	0.086
Beta-2	(491.1 ± 169.4)	1939.6 ± 939.6	2174.0 ± 1151.2	2366.0 ± 942.7	2522.8 ± 1196.3	
PaO2, mmHg						
Control	(569.8 ± 28.3)	349.4 ± 102.0	213.0 ± 160.6	139.2 ± 133.0	112.8 ± 87.3	< 0.001
Beta-2	(623.6 ± 42.3)	625.2 ± 55.1	516.6 ± 161.1	537.0 ± 131.7	526.8 ± 130.2	
PaCO2, mmHg						
Control	(17.2 ± 1.9)	31.9 ± 6.3	39.1 ± 4.5	48.5 ± 9.1	47.3 ± 6.0	0.24
Beta-2	(20.9 ± 2.3)	32.1 ± 1.3	39.3 ± 6.3	39.5 ± 9.1	39.0 ± 8.6	
Dynamic compliance, ml/cmH ₂ O						
Control	(19.4 ± 1.1)	12.9 ± 1.3	13.8 ± 1.6	13.8 ± 1.6	13.6 ± 1.0	0.013
Beta-2	(18.4 ± 1.8)	19.9 ± 2.6	18.7 ± 3.6	17.3 ± 3.1	16.9 ± 3.1	

OP = operation; AoP = aortic pressure; PAP = pulmonary artery pressure; CVP = central venous pressure; LAP = left atrial pressure; PVR = pulmonary vascular resistance. The data are shown as the means ± SD. P-values are for the differences between groups determined by a repeated measures analysis of variance between 75 and 240 min.

Table 2. Energy levels in normal lungs (A) and in recipients 240 min after reperfusion (B).

A

Energy levels	Normal lung
ATP (nmol/mg·dw)	4.20 ± 0.57 ^a
Energy charge ^b	0.79 ± 0.02
TAN ^c (nmol/mg·dw)	6.21 ± 0.59

B

Energy levels	Control group	Beta-2 group	<i>P</i> -value
ATP	2.21 ± 0.71	4.42 ± 1.35	0.012
Energy charge	0.85 ± 0.05	0.87 ± 0.03	0.48
TAN	2.83 ± 0.86	5.43 ± 1.51	0.027

^aThe data are presented as the means ± SD and were analyzed with unpaired *t*-tests.

^bEnergy charge = (ATP + 0.5ATP) / (ATP + ADP + AMP).

^cTAN = ATP + ADP + AMP.

ADP = adenosine diphosphate; AMP = adenosine monophosphate; ATP = adenosine triphosphate; TAN = total adenosine nucleotides.